THE ASSOCIATION OF VIRULENT *VIBRIO* SPP. BACTERIA ON GAFFTOPSAIL AND HARDHEAD CATFISH IN GALVESTON BAY

A Thesis

by

LESLIE DEANNE GILBERT

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2010

Major Subject: Wildlife and Fisheries Sciences

The Association of Virulent *Vibrio* spp. Bacteria on Gafftopsail and Hardhead Catfish in Galveston Bay

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Approved by:

Chair of Committee,	Robin Brinkmeyer
Committee Members,	John Schwarz
	Delbert Gatlin III
Head of Department,	Thomas E. Lacher Jr

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ABSTRACT

The Association of Virulent *Vibrio* Spp. on Gafftopsail and Hardhead Catfish in Galveston Bay. (August 2010)

Leslie Deanne Gilbert, B.S., Texas A&M University at Galveston

Chair of Advisory Committee: Dr. Robin Brinkmeyer

Vibrio vulnificus (Vv) and *V. parahaemolyticus (Vp)* are gram negative, halophilic bacteria that occur naturally in estuarine waters of Galveston Bay. Both bacteria have the potential to cause infections in humans either via consumption or direct contact. Finfish are a potential vector for these bacteria. Previous work by Brinkmeyer determined that these bacteria are present on the benthic dwelling catfish, *Ariopsis felis* and *Bagre marinus*, using a conventional microbial method. The present work focused on using Quantitative Polymerase Chain Reaction (QPCR) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) to not only determine presence of these bacteria, but also to quantify them and look at community structure.

QPCR was able to detect bacteria presence in 34%, 31.6%, and 0% for *V.vulnificus, V.parahaemolyticus.* thermolabile hemolysin (*tlh*) and thermostable direct hemolysin (*tdh*) genes, respectively. Statistical analysis of the QPCR results found that there was no significant difference between the length of fish, location of catch or species of fish in relation to the abundance of bacteria.

T-RFLP was able to detect the presence of bacteria in approximately 70% of the samples surveyed. Bands produced from T-RFLP were able to be grouped into five different ranges. The most frequently occurring band fell in the range of 213-219 base pairs, and the most common number of bands per sample was 1 band.

This study found that both QPCR and T-RFLP were better assays than conventional microbial methods for detecting the presence of *V. vulnificus* and *V. parahaemolyticus* on catfish fins. QPCR proved to be the most rapid detection method. Based on this study, it was determined that these *Vibrio* spp. bacteria have some type of relationship with *A. felis* and *B. marinus*. This information may be useful to the medical community for determining when there is a greater risk of infection via catfish puncture wounds.

DEDICATION

To my mom, dad: Thank you for everything you have given to help get me to where I am today. None of this would have been possible without you and your love and encouragement.

To my brother Kyle, my real American hero.

To Margaret “Granny” Roberts and J.E. Bob “PawPaw” Roberts: Thank you for always encouraging me to continue my education and giving me the opportunity to do so.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Robin Brinkmeyer, and my committee members, Dr. John Schwarz and Dr. Delbert Gatlin III, for their support and guidance throughout my research project.

Many thanks go to my colleagues in the Coastal Health and Estuarine Microbiology Lab and Seafood Safety Lab for their help and encouragement. In particular I would like to thank Mona Hochman for helping with the cultivation aspect; Nicole Towers for helping with the fingerprinting; and Tara Hans for helping with quantification. Also, special thanks to Elizabeth Neyland for help troubleshooting and Patty Edwards for help with statistical analysis.

Finally, special thanks to the Texas Parks and Wildlife Dickinson Marine Lab and the local Galveston shrimpers who helped me collect samples.

NOMENCLATURE

ANOVA	Analysis of Variance
GSC	Galveston Ship Channel
QPCR	Quantitative real-time Polymerase Chain Reaction
TRFLP	Terminal Restriction Fragment Length Polymorphism
VBNC	Viable But Non-culturable

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CHAPTER I

INTRODUCTION

Introduction

This study examined the potential of the saltwater catfishes, *Ariopsis felis* and *Bagre marinus* as vectors for virulent *Vibrio parahaemolyticus* and *Vibrio vulnificus* infections in humans. The ecology, i.e. salinity and seasonality (Joseph et al., 1982; Daniels et al., 2000; Borenstein and Kerdel, 2003), of these vibrios has been well studied and infection typically occurs through ingestion of seafood, such as oysters. However, non-foodborne *Vibrio* infections (NFVI) can also occur. Dechet et al. (2008) surveyed the Centers for Disease Control and Prevention's Cholera and Other *Vibrio* Illness Surveillance System (COVIS) reports from 1997 to 2006 and found that at least 21% of *Vibrio*-caused illnesses were non-foodborne, with 68% of these being *V. vulnificus* having a mortality rate of 17% and 10% requiring amputation; 19% of NFVIs were caused by *V. parahaemolyticus.* Cutaneous and soft tissue infections result from exposure of previously acquired wounds to seawater or marine-related injury such as lacerations from broken shells and by puncture wounds from crabs, shrimp and fish finning (Oliver, 2005; Dechet et al., 2008). Unfortunately, COVIS records do not always provide specific details of finfishes and other marine organisms that are the source of infection, but a review of scientific literature describing *V. vulnificus* and *V. parahaemolyticus* wound infections found punctures from fish finning to soft tissue, in

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This thesis follows the style of Limnology and Oceanography.

particular from catfish spines to be among the most virulent of cases (Bonner et al., 1983; Baack et al., 1991; Midani et al., 1994; Klontz et al., 1998; Calif et al., 2003; Tsai et al., 2004; Chein-Chang et al., 2007; Ralph and Currie 2007).

Vibrio parahaemolyticus

Vibrio parahaemolyticus is a gram-negative, halophilic bacterium (Daniels et al., 2000). It is the most common *Vibrio* species that is isolated in humans and the most frequent cause of *Vibrio* related gastroenteritis (Daniels et al., 2000).

Vibrio parahaemolyticus can grow at temperatures ranging from 9 to 44 °C with an optimum range of 35 to 37 °C (Joseph et al., 1982). Growth cycles depend on the season, with higher numbers seen in the summer and fall and lowest numbers in the winter (Joseph et al., 1982). In tropical climates, isolation of *V. parahaemolyticus* occurs throughout the year (Novotny et al., 2004).

It has a widespread occurrence, mainly being found in estuarine environments and rarely seen in the pelagic regions of the open ocean (Joseph et al., 1982). *Vibrio parahaemolyticus* has been found in association with many different higher organisms, including 30 different species of fish (Joseph et al., 1982). However, isolation from fish is not as frequent as isolation from filter feeding invertebrates (Joseph et al., 1982).

In a large enough quantity, *V. parahaemolyticus* can cause acute gastroenteritis in humans (Joseph et al., 1982; Novotny et al., 2004). Generally these infections are self limiting (Novotny et al., 2004; Blackwell and Oliver, 2008). However many cases require hospitalization and in rare cases can cause septicemia (Novotny et al., 2004).

Wound infections pose a higher risk of death and are recognized as a significant cause of life-threatening infections (Colmer-Hamood, 2007).

Vibrio vulnificus

Vibrio vulnificus is also a gram-negative, halophilic bacterium (Borenstein and Kerdel, 2003). It is a virulent human pathogen that is found worldwide and is considered to be one of the most invasive and rapidly lethal human pathogens (Calif et al., 2003). Optimum temperatures for growth are those above 20 °C, with optimum salinity between 7 and 16 ppt (Borenstein and Kerdel, 2003).

Infections occur either by consumption or direct contact via contamination of wounds (Calif et al., 2003). These infections can result in gasteroenteritis, wound infections and primary septicemia (Calif et al., 2003). Infections from *V. vulnificus* are more common in patients with liver disease or who are immuno-compromised (Borenstein and Kerdel, 2003).

Epidemiology files from the Galveston area have shown *V. vulnificus* and *V. parahaemolyticus* infections arise as a result of stab wounds from hard head (*Ariopsis felis*) and gafftopsail (*Bagre marinus*) catfish. In a study done by DePaola et al. (1994), *V. vulnificus* has been found in abundance among estuarine fish in the US Gulf coast area. Because of the mobility of finfish over filter-feeding invertebrates, the presence of *V. vulnificus* on these finfish can have serious ecological and public health implications (DePaola et al., 1994). Finfish, such as *A. felis* and *B. marinus*, can potentially transport these bacteria to more optimal locations for growth (DePaola et al., 1994).

Viable but Non-culturable State

Many bacteria have the ability to enter into a viable but non-culturable (VBNC) state when conditions become adverse (Oliver, 2005). In this state, they will fail to grow on bacteriological media that they normally would grow on (Oliver, 2005). According to Oliver (2005), typically when bacteria are subjected to environmental stresses there is a decline in the number of colony forming units (CFU), but the 'total cell counts' remain consistent. However, to determine if cells are still alive, an assay must be done to determine the 'viability count' (Oliver, 2005). In all cases, these assays show that some type of metabolic process is still going on, even if cells are not growing on media (Oliver, 2005). The VBNC state does differ from the 'starvation survival' state, in that during the starvation survival state cells are still culturable (Oliver, 2005). Stresses that can induce this state include the following: starvation, temperatures outside the normal growth range, elevated osmotic concentrations, elevated oxygen concentrations or exposure to white light (Oliver, 2005).

Both *V. vulnificus* and *V. parahaemolyticus* have the ability to enter into this VBNC state (Oliver, 2005). Wolf and Oliver (1992) found that entry of *V. vulnificus* to this VBNC state is highly dependent on temperature. For *V. parahaemolyticus*, Jiang and Chai (1996) found that cells will enter into a VBNC state when exposed to low temperature and nutrient starvation.

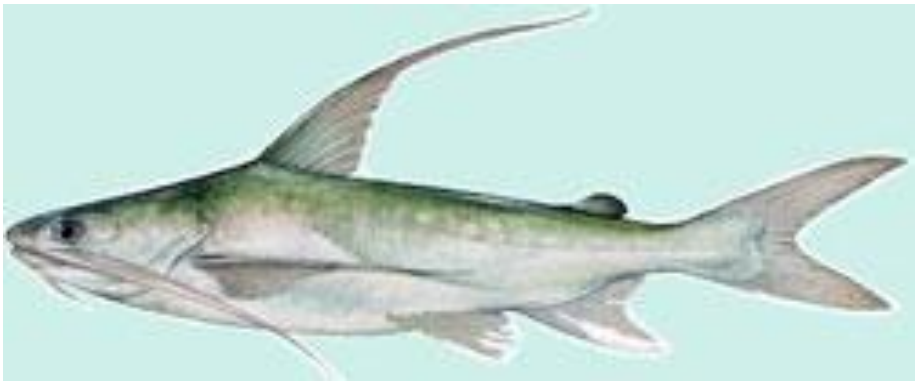
Catfish Specimens

Ariopsis felis (hardhead catfish; fig. I.1A) and *Bagre marinus* (gafftopsail catfish fig. I.1B) are both found in and around Galveston bay. *Ariopsis felis* and *B. marinus* are opportunistic feeders, feeding over mud and submerged sand flats. Both have venomous spines in the dorsal and pectoral fins (fig. I.1C) that can inflict deep tissue wounds.

Studies have shown that both species tend to favor higher temperatures and salinities. During warmer months both catfish prefer inshore areas and will migrate offshore as temperatures decline. Optimal temperatures for both catfish species are 25 °C and higher; however, *A. felis* tends to avoid temperatures exceeding 37 °C. Adult hardhead catfish can be found in salinities ranging from 0 – 40 ppt. While gafftopsail catfish have been found in freshwater, they tend to prefer salinities of 5 – 30 ppt. These temperature and salinity ranges correspond with those favored by the bacteria *V. parahaemolyticus* and *V. vulnificus*.

A

B



C

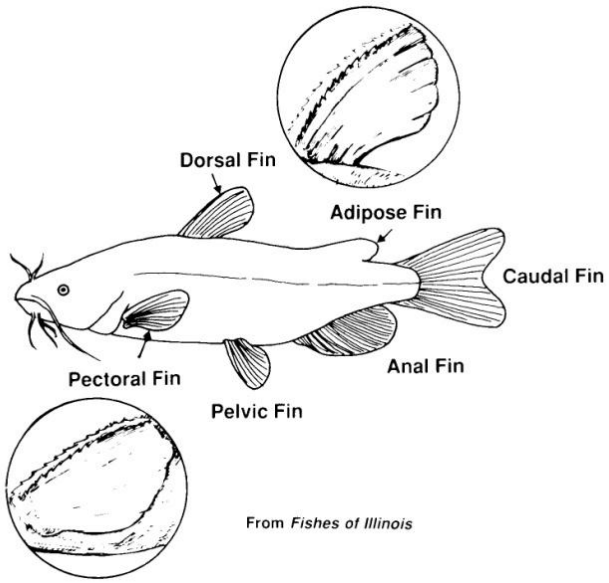


Figure I.1. Hardhead catfish (A) (The Jump, 2010), gafftopsail catfish (B) (Smithsonian Marine Station at Fort Pierce, 2010), and anatomy of the catfish indicating the spines in the dorsal and pectoral fins (C) (Muncy and Wingo, 1983).

Study Area

Galveston Bay is the largest estuary in the Texas, located on the Southeast Texas coast. Seawater comes in via the Gulf of Mexico, while freshwater enters from the Trinity and San Jacinto Rivers. The Galveston Bay system is composed of four major sub-bays: Galveston, Trinity, East and West Bays (Fig I. 2). Galveston Bay lies adjacent to Houston, the largest city in Texas and 4th largest city in the United States. Houston is also home to the Port of Houston. The Port of Houston is the largest port in the U.S., based on foreign tonnage, second based on domestic tonnage and the 6th largest in the world (Galveston Bay, 2009).

All four of the major sub bays were surveyed during this project, with the greatest amount of samples coming from Galveston Bay and East Bay. Locations of the Texas Parks and Wildlife sites can be seen on Figure I.3. The location of the fish obtained from shrimpers was approximated as samples were collected after they had come back into the docks. This is noted as the Galveston Ship Channel (GSC).

Figure I. 2. Map of Galveston Bay system.
(Galveston Bay Estuary Program, 2010)

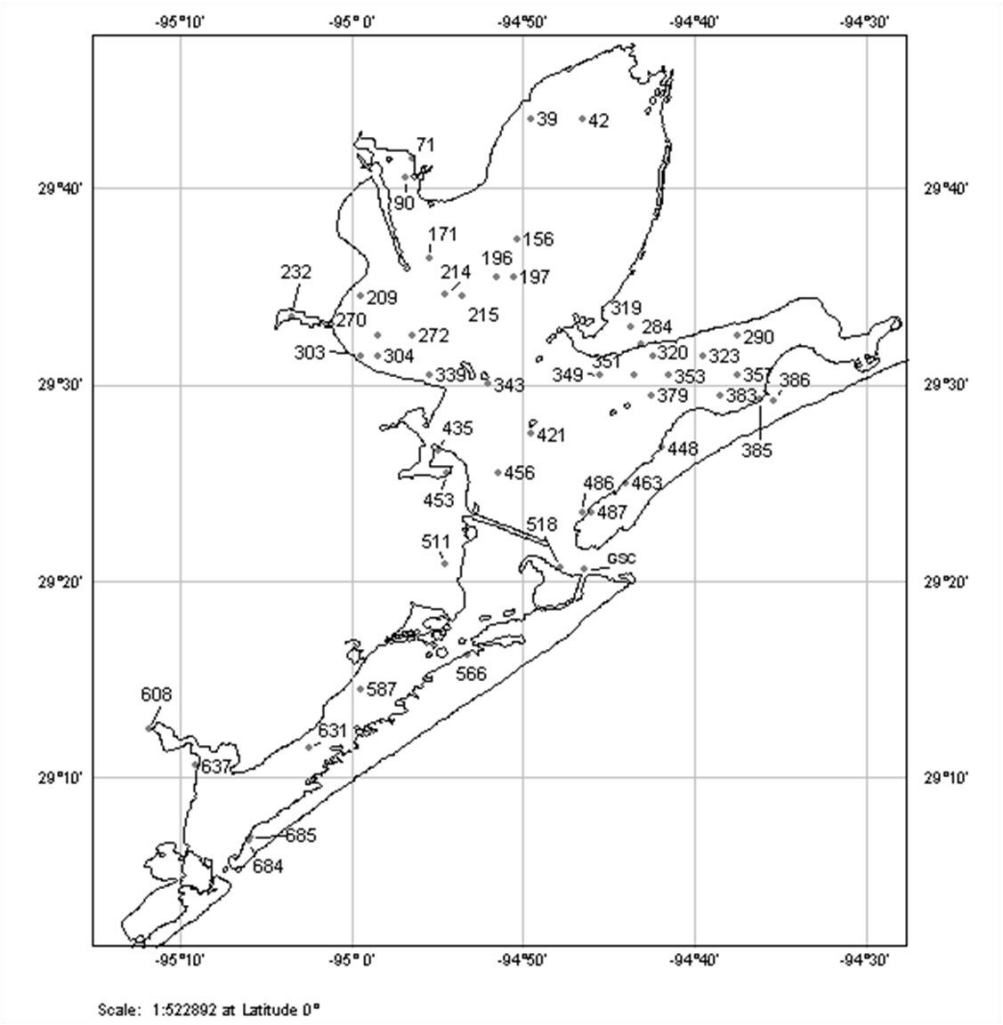


Figure I. 3. Sample collection sites.

Research Objectives

The objective of this study was to characterize populations of *V. vulnificus* and *V. parahaemolyticus* occurring on the benthic-dwelling fishes *A. felis* and *B. marinus* in Galveston Bay. A previous study in the Brinkmeyer lab (Brinkmeyer et al. in prep) determined that 50% and 94% of catfish in Galveston Bay tested positive for *V.*

vulnificus and *V. parahaemolyticus*, respectively. Of the sites tested, 35% and 4% were found to have an 80% or greater occurrence of *V. vulnificus* and pathogenic *V. parahaemolyticus*, respectively. Quantitative PCR was used to enumerate *V. vulnificus* and *V. parahaemolyticus* on samples that tested positive and DNA fingerprinting was used to look at community structure of all *Vibrio* spp. bacteria present.

Hypothesis 1: Benthic-dwelling fishes, such as *A. felis* and *B. marinus*, are important vectors for virulent *Vibrio vulnificus* and *Vibrio parahaemolyticus*.

Hypothesis 2: Quantitative Polymerase Chain Reaction and Terminal Restriction Fragment Length Polymorphism will be better assays for determining presence or absence of *Vibrio vulnificus* and *Vibrio parahaemolyticus* than more conventional microbial methods.

CHAPTER II

QUANTIFICATION OF *VIBRIO* SPP. BACTERIA ON GAFFTOPSAIL AND HARDHEAD CATFISH IN GALVESTON BAY

Introduction

In a previous study (Brinkmeyer et al. in prep), 50% and 94% of catfish (combined species) in Galveston Bay tested positive for *V. vulnificus* and *V. parahaemolyticus*, respectively. Of the sites tested, 35% and 4% were found to have an 80% or greater occurrence of *V. vulnificus* and pathogenic *V. parahaemolyticus*, respectively. The gene probe method used by the Seafood Safety Lab at TAMUG to screen oysters for presence of the cytolysin gene in *V. vulnificus* (DePaola et al., 1997) and *tlh* and *tdh* genes in *V. parahaemolyticus* (DePaola et al., 2003) was used to screen the catfish for presence or absence but not quantification of these virulent *Vibrios*. An estimate of the amounts of *V. vulnificus* and *V. parahaemolyticus* that occur on the fins and spines is important to access the potential of the catfish as a vector for human infection. The infective dose for ingestion for both species is 10^6 but can be as low as 10^2 for predisposed persons (Ohio Department of Health, 2009). To my knowledge, there are currently no data regarding the infection doses for wound exposure to *V. vulnificus* and *V. parahaemolyticus*.

A review of scientific literature describing *V. vulnificus* and *V. parahaemolyticus* wound infections found punctures from fish finning to soft tissues, in particular from catfish spines, to be among the most virulent of cases (Bonner et al., 1983; Baack et al.,

1991; Midani et al., 1994; Klontz et al., 1998; Calif et al., 2003; Tsai et al., 2004; Change et al., 2007; Ralph and Currie 2007). Epidemiology files from the Galveston area have shown *V. vulnificus* and *V. parahaemolyticus* infections arise as a result of stab wounds from hard head (*Ariopsis felis*) and gafftopsail (*Bagre marinus*) catfish. Both have venomous spines in the dorsal and pectoral fins that can inflict deep tissue wounds.

In this chapter, *V. vulnificus* and *V. parahaemolyticus* on the spines and fins of *Ariopsis felis* and *Bagre marinus* were enumerated with quantitative PCR assays that target toxicity genes. These quantitative data were also tested to determine if the associations of these bacteria are related to fish size (length), location in Galveston Bay, and catfish species to provide insights about the ecology of *V. vulnificus* and *V. parahaemolyticus* and predictability of their occurrence.

Materials and Methods

Sample Collection

In the summer of 2006, hardhead (*A. felis*) and gafftopsail (*B. marinus*) catfish were collected with the assistance of the Texas Parks and Wildlife Dickinson Marine Lab in conjunction with their fish population studies and local Galveston shrimpers. Fish were also collected in the fall of 2006 through the Texas Parks and Wildlife Dickinson Marine Lab gill net program. Fish were transported to the lab on ice where spines and fins were removed for bacterial culturing. A total of 375 fish were obtained for this study. Fish were also measured for total length to the nearest half inch, later converted to the nearest centimeter.

Processing of Samples

Fins and spines were removed using sterile forceps and bone cutting pliers. The dorsal fin together with its spine and one of the pectoral fins together with its spine from each individual fish were transferred to 15-ml conical tubes containing 5 ml of alkaline peptone water (APW) for 24 h cultivation at 35 °C. All, or part, or the second pectoral fin and spine from each fish was transferred to 2.0-ml tubes and frozen at -20 °C for later DNA extraction. The cultivated *V. vulnificus* and *V. parahaemolyticus* bacteria were isolated using media specific for each species and then further analyzed for toxicity genes, i.e. vvh and *tdh*/*tlh* respectively, using alkaline phosphatase labeled DNA probes as described Wright et al. (1993) and Drake et al. (2007). Bacteria were grown on selective media for isolation over a period of three days. Isolated colonies were then affixed to Whatman filters via microwave. Filters were then put through a series of washes for neutralization and to remove any background alkaline phosphatase activity. After being washed, the filters were then hybridized with alkaline phosphatase probes and chemically visualized with NBT-BCIP.

Extraction of Nucleic Acids

Bacterial DNA from pectoral fins stored at -20 °C was extracted using a Cetyltrimethyl Ammonium Bromide (CTAB)/chloroform-isoamyl alcohol method (Doyle and Doyle, 1990). In short, fins were incubated in a 3% CTAB buffer (500 µl) at 65°C for 30 min. Chloroform-isoamyl alcohol (500 µl) was added to the sample, vortexed, and then centrifuged to separate the DNA from the cell material. The aqueous

top layer was transferred to a clean tube and 100% cold isopropyl alcohol (60% volume) was added to precipitate the DNA. DNA was harvested by centrifugation, supernatant discarded, pellet washed with cold 80% ethanol and air dried. DNA was re-suspended in 200 µl low Tris (LT) buffer (dilute Tris-Acetate-EDTA, TAE, buffer), analyzed for concentration and purity with a Nanodrop spectrophotometer, and stored at -20 °C for later processing.

Quantitative PCR

Quantitative PCR (QPCR) is a means by which to measure template DNA quantitatively (Lie and Petropoulos, 2002). This can be done with real time detection by using fluorescing probes. Generally, these probes have two different fluorophores: a reporter on the 5' end and a quencher in the middle or on the 3' end. This non-extendible probe will anneal itself to a target sequence that falls between the two primer binding site (Heid et al., 1996; Lie and Petropoulos, 2002). During the elongation phase of a cycle, the probe will be cleaved by the Taq polymerase releasing the reporter from the probe thereby increasing the emission intensity (Lie and Petropoulos, 2002). The cycle at which the emission intensity surpasses the baseline intensity is known as the threshold cycle. This cycle is inversely proportional to the amount of target concentration, meaning the greater the target concentration the fewer the cycles needed to surpass the baseline intensity (Lie and Petropoulos, 2002).

Extracted DNA from the fin samples was analyzed using QPCR to enumerate the number of colony forming units (CFU) present per milliliter of sample. QPCR was run

on environmental samples (second pectoral fin) to determine abundance of *V. parahaemolyticus* (*tlh* gene containing strains), *V. vulnificus, and V. cholerae*, which also occurs naturally in Galveston Bay, was enumerated with QPCR as a follow up analysis from a previous study. The primers and probes that were used are listed in Table II.1.

Table II.1. QPCR primers and probes.

Primer Name	Sequence	Probe	Quencher	Reference
V.v.				
vvh				Panicker and Bej 2004
Forward	TTCCAACTTCAAACCGAACTATGA	SYBR Green		
Reverse	ATTCCAGTCGATGCGAATACGTTG			
V.p.				
tdh				Nordstrom et al. 2007
Forward	TCCCTTTTCCTGCCCCC			
Reverse	CGCTGCCATTGTATAGTCTTTATC			
Probe	TGACATCCTACATGACTGTG	5' FAM to 3'	MGBNFQ	
tlh				Nordstrom et al. 2007
Forward	ACTCAACACAAGAAGAGATCGACAA			
Reverse	GATGAGCGGTTGATGTCCAA			
Probe	CGCTCGCGTTCACGAAACCGT	5' TxRED to 3	BHQ2	
V.c.				
ctx A				Blackstone et al. 2006
Forward	TTTGTTAGGCACGATGATGGAT			
Reverse	ACCAGACAATATAGTTTGACCCACTAAG			
Probe	TGTTTCCACCTCAATTAGTTTGAGAAC	5' FAM to 3'	BHQ1	

Master mixes were made in a Purifier Biological Safety Cabinet (Labconco), where all components except for template and internal control (IC) DNA were added to a clean 1.5-mL microcentrifuge tube. In a separate dead air safety cabinet (C.B.S.

Scientific Co.), the IC DNA was added to the master mix, if necessary, the master mix was aliquoted out into SmartCycler tubes and template DNA added. IC DNA is non-homologous to any bacteria in GenBank and serves to ensure that the PCR is working without doing replicates. For each run, a positive control and negative control were run.

QPCR was then run with the SmartCycler 2.0d (Cepheid) using the protocol of Nordstrom et al. (2007) for *V. parahaemolyticus*. This program was modified from the original multiplex to single reactions as it was found that the *V. parahaemolyticus tlh* strain would be preferentially amplified over any *V. parahaemolyticus tdh* strain that might also be found in the sample. For *V. cholerae* the protocol followed was of Blackstone et al. (2006) and the protocol of Panicker and Bej (2004) was used for *V. vulnificus*.

Data were compared to a standard curve created for each of the bacteria. Controls were grown up overnight in 3 mL of alkaline peptone water (APW) in a 35 °C shaking water bath. After incubation, 1 mL of APW was aliquoted for a serial dilution to do plate counts and 1 mL of APW was aliquoted for extraction and QPCR. Serial dilution for the plate counts was done by adding 100 µL of sample into 900 µL of PBS initially and then 100 µL of each dilution into the next going from 10^0 to 10^{-6}. Dilutions were plated out onto Luria Broth (LB) agar plated with 1% sodium chloride, incubated overnight at 35 °C and counted the following day. The other 1 mL was extracted using the same CTAB/chloroform-isoamyl extraction method (Doyle and Doyle, 1990) for consistency of methods. Once extracted, the DNA was serially diluted by adding 20 µL of sample into 180 µL of LT buffer. To create the standard curve, dilutions were run through the

QPCR protocol mentioned above and then assigned the corresponding CFU/ml value determined by plate counts.

Results

Vibrio vulnificus

A total of 250 samples were screened for *V. vulnificus* presence only. Of the samples tested, 85, or 34%, yielded positive results from the QPCR assay. Table II.2. shows the number of samples that originally produced positive and negative results from gene probe and how they compared to QPCR.

Table II.2. Comparison of gene probe analysis and QPCR analysis for *V. vulnificus.*

Gene Probe Positive			Gene Probe Negative		
QPCR Positive	QPCR Negative	Total	QPCR Positive	QPCR Negative	Total
46	65	111	39	100	139

Of the samples that had initially given a positive result with gene probe, only 41% also yielded a positive result for QPCR. For the samples that had a negative result with gene probe initially, about 28% gave a positive result with QPCR. It is important to note that the gene probe method was used on samples that were incubated for 24 h in APW a growth media that selects for *Vibrio* bacteria; whereas, the samples used for QPCR were not enriched.

The quantity of *V. vulnificus* ranged from 0 to 63.6 CFU/ml with the exception of an outlier, a fin having 52,565 CFU/ml. The highest quantities were associated with fishes having 38-51 centimeters total length. Trinity Bay that receives freshwater

directly from the Trinity River had the highest amount of *V. vulnificus*, and hardhead catfish were the predominant species (fig. II.1. A, B, C).

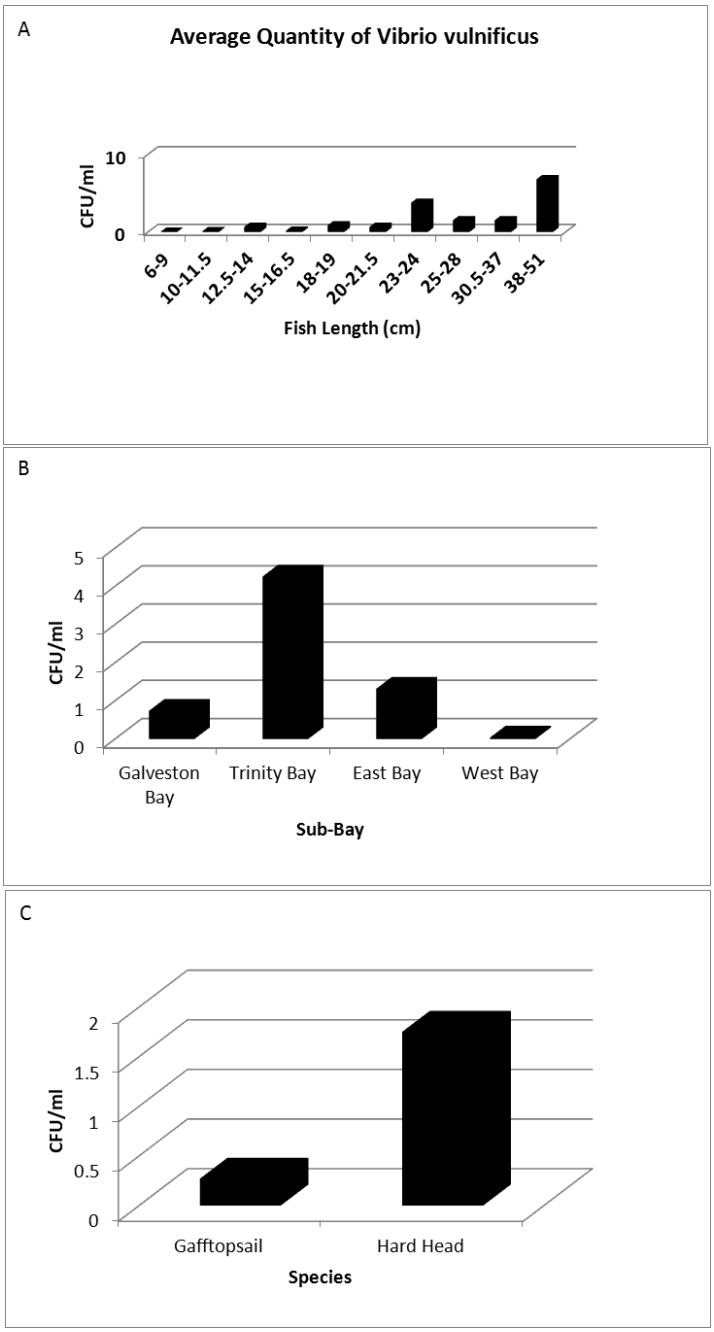


Figure II.1. Distribution of *V. vulnificus* by A) fish length, B) sub-bay, and C) species of catfish.

Linear regressions were calculated using the averages for each parameter set tested: length of fish (in centimeters), location of collection (by sub-bay) and species. All parameters were grouped into ranges respective to what they are showing. Numerical values were given to location of collection and species. Lengths were consolidated for a better comparison of averages using linear regressions and for better group comparisons in SPSS. Ranges can be found in Table II.3.

Table II.3. Values for linear regression comparison.

	Length Range	Sub-bay	Species
1	6-12.5 cm	Galveston Bay	Gafftopsail Catfish
2	12.5-18 cm	Trinity Bay	Hardhead Catfish
3	18-23 cm	East Bay	
4	23-28 cm	West Bay	
5	28-51 cm		

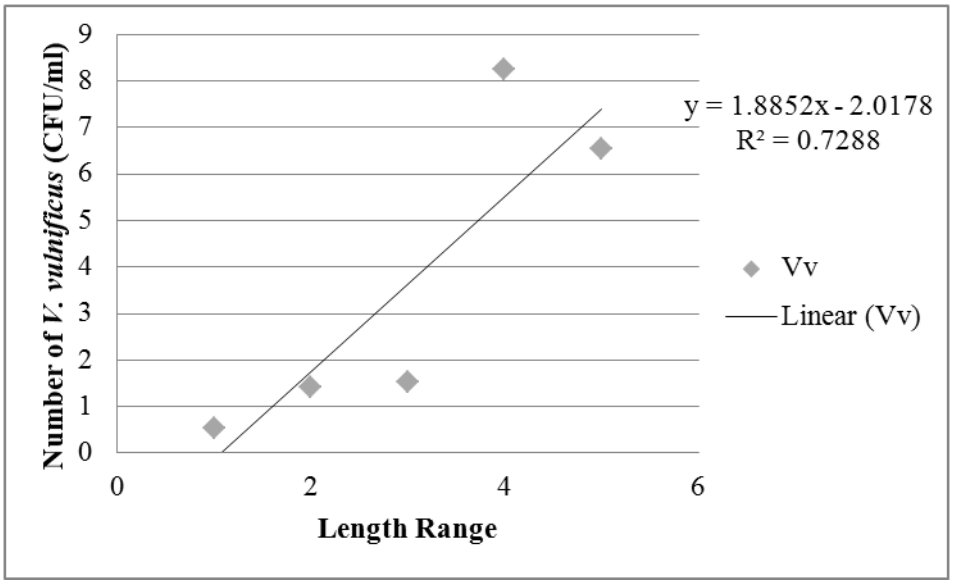


Figure II.2. Correlation of *V. vulnificus* abundance versus length of fish.

An R^2 value of 0.7288 shows a moderate relationship between the average number of bacteria per size group of fish and the length of fish (fig. II.2.).

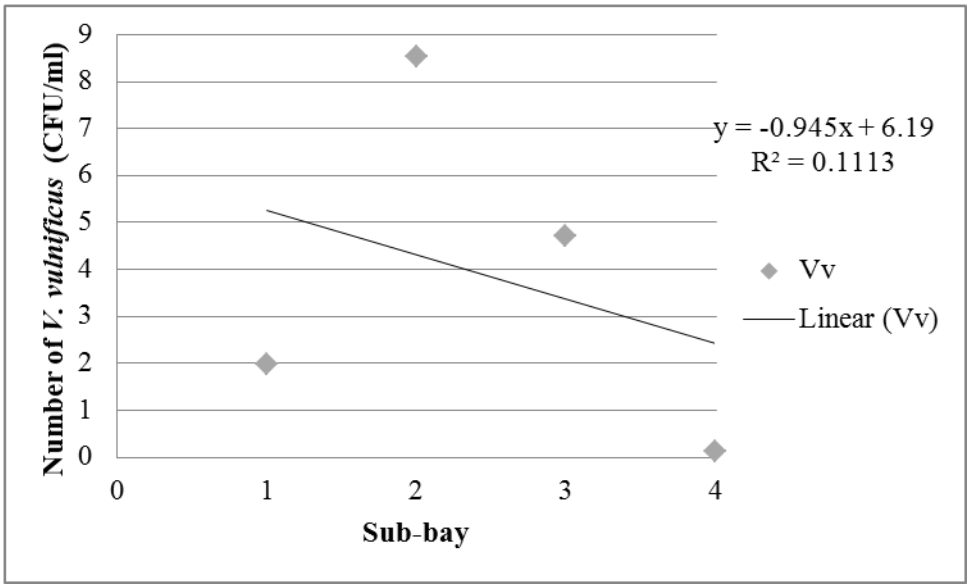


Figure II.3. Correlation of *V. vulnificus* abundance versus location of fish.

An R^2 value of 0.1113 shows a very minimal relationship between the average number of bacteria per sub-bay and the location (fig. II.3.).

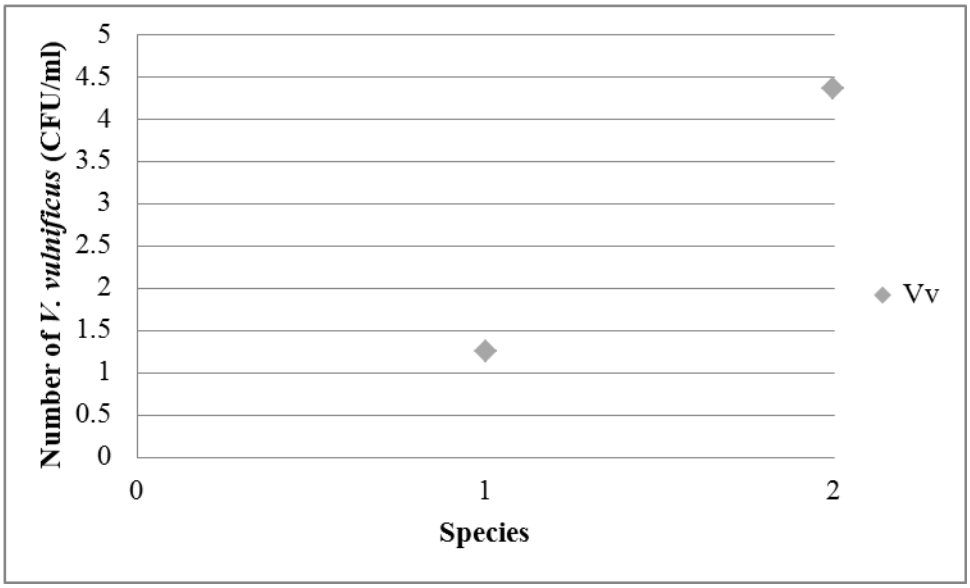


Figure II.4. Correlation of *V. vulnificus* abundance versus species of fish.

A linear regression trendline was not added to figure II.4. as there were only two data points for comparison.

Next, more sensitive tests were conducted using SPSS to determine if any of these relationships were significant. First, normal distribution of data was determined through a Kolmogorov-Smirnov test.

Table II.4. Kolmogorov-Smirnov results for *V. vulnificus*

	V. vulnificus
Kolmogorov-Smirnov Z	3.649
Asymp. Sig. (2 tailed)	0.000

A p value of less than 0.05 indicated that the data was normally distributed for the *V. vulnificus* data points (table II.4.).

Once it was determined that the data were normally distributed, analysis of variance (ANOVA) was performed to see if any relationship was significantly different between the number of bacteria found and species, length or location found.

Post hoc tests were also run, if the ANOVA p value was less than 0.05, indicating a significant difference. These tests were Tukey's, for equal variance assumed, and Tamhane's T2, for unequal variance assumed. If there was a significant difference, these tests would indicate which variable(s) was significantly different from the others.

Table II.5. ANOVA results for quantity of *V. vulnificus* versus length of fish.

	Sum of Squares	df	Mean Square	F	p
Between Groups	670.617	4	167.654	1.598	0.183
Within Groups	8182.970	78	104.910		
Total	8853.587	82			

The p value for the number of *V. vulnificus* versus length was 0.183. This is greater than a value of 0.05, which means that there is no difference in the number of bacteria based on the length of the fish (table II.5.).

Table II.6. ANOVA results for quantity of *V. vulnificus* versus location of fish.

	Sum of Squares	df	Mean Square	F	p
Between Groups	443.950	3	147.983	1.390	0.252
Within Groups	8409.637	79	106.451		
Total	8853.587	82			

The p value for the number of *V. vulnificus* versus the location of the fish was 0.252. This value is greater than 0.05, which indicates no significant difference in the number of bacteria based on the location of the fish (table II.6.).

Table II.7. ANOVA results for the quantity of *V. vulnificus* versus species of fish.

	Sum of Squares	df	Mean Square	F	p
Between Groups	146.413	1	146.413	1.362	0.247
Within Groups	8707.174	81	107.496		
Total	8853.587	82			

The p value for the quantity of *V. vulnificus* versus the species of fish was 0.247. This value was greater than 0.05, indicating no significant difference in the quantity of *V. vulnificus* based on the species of fish (table II.7.).

Vibrio parahaemolyticus

A total of 250 samples were also screened for *V. parahaemolyticus* presence, both the *tlh* and *tdh* strains. Of the samples tested for *V. parahaemolyticus tlh* strain, 79 or 31.6%, yielded positive results from the QPCR assay. For *V. parahaemolyticus tdh*

strain, 0 or 0%, yielded a positive result via QPCR assay. Listed in table II.8. and II.9. are comparisons of samples initially run through gene probe, and later QPCR.

Table II.8. Comparison of gene probe analysis and QPCR analysis for *V. parahaemolyticus tlh*.

Gene Probe Positive			Gene Probe Negative		
QPCR Positive	QPCR Negative	Total	QPCR Positive	QPCR Negative	Total
73	162	235	6	9	15

Table II.9. Comparison of gene probe analysis and QPCR analysis for *V.parahaemolyticus tdh*.

Gene Probe Positive			Gene Probe Negative		
QPCR Positive	QPCR Negative	Total	QPCR Positive	QPCR Negative	Total
0	39	39	0	211	211

Of the samples that initially yielded a positive result with gene probe for *V. parahaemolyticus tlh*, only 31% also tested positive via QPCR. For the samples that had first given a negative result via gene probe, 40% gave a positive result via QPCR. Again, it is important to note that the gene probe method was use on samples that were incubated for 24 h in APW, a growth media that selects for *Vibrio* bacteria; whereas, those used for QPCR were not.

The quantity of *V. parahaemolytics* (*tlh*) ranged from 0 to 4320 CFU/ml. The highest quantities were associated with fishes greater than 23 centimeters total length.

Similar to *V. vulnificus*, *V. parahaemolyticus* were predominantly associated with catfish collected in Trinity Bay as well as with hardhead catfish (fig. II.5 A, B, C).

As with *V. vulnificus*, linear regressions were estimated for *V. parahaemolyticus* against fish length, location of collection, and species of fish. Group designations remain the same as with *V. vulnificus* and can be found in table II.3.

An R^2 value of 0.2562 shows a weak relationship between the number of *V. parahaemolyticus tlh* bacteria and the length of fish (fig. II.6). An R^2 value of 0.0289 shows a very minimal relationship between the quantity of bacteria and the location of the fish (fig. II.7.). A linear regression trendline was not added to Figure II.8. as there were only two data points for comparison.

As with *V.vulnificus.*, more sensitive tests were done using SPSS to see if any of these relationships were significant for *V. parahaemolyticus tlh*. First, normal distribution of data was determined through a Kolmogorov-Smirnov test.

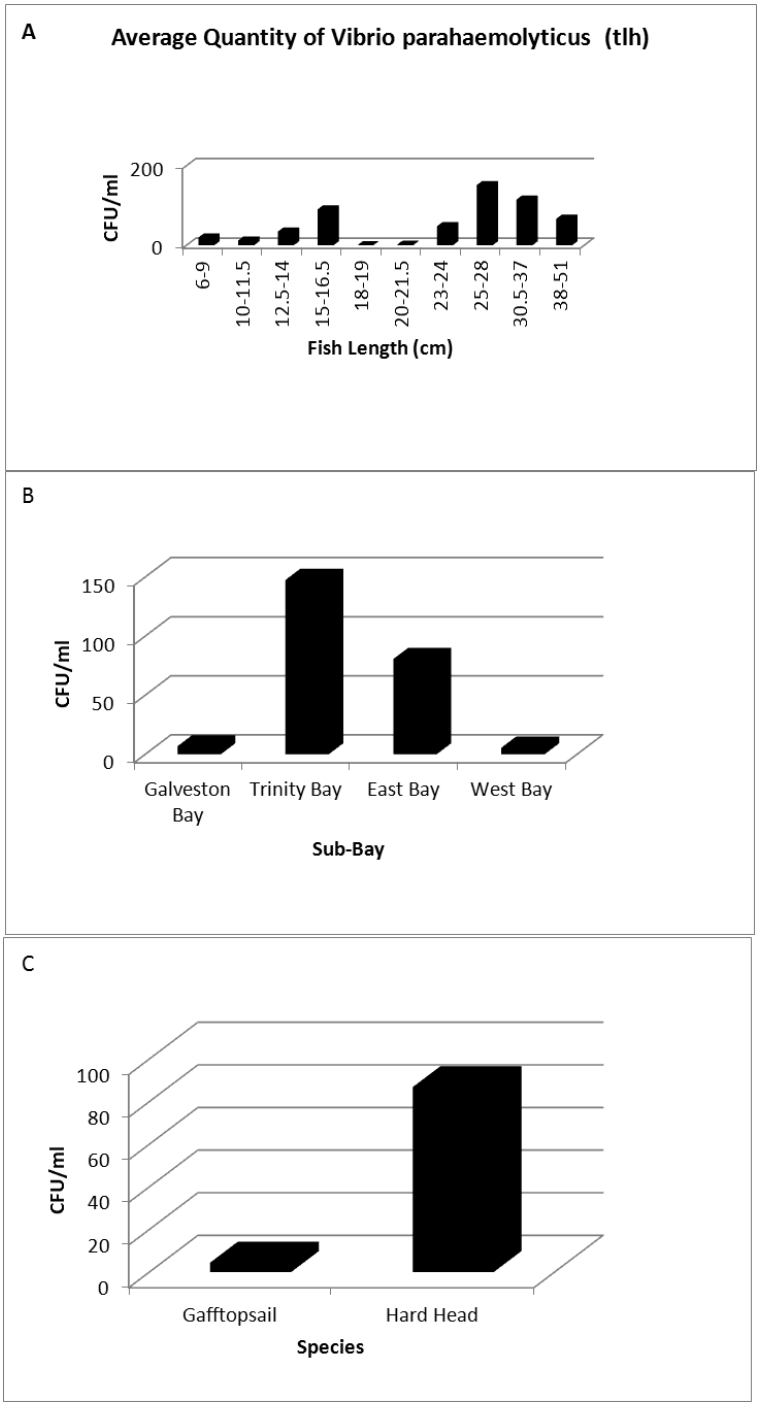


Figure II.5. Distribution of *V. parahaemolyticus tlh* by A) fish length, B) sub-bay, and C) species of catfish

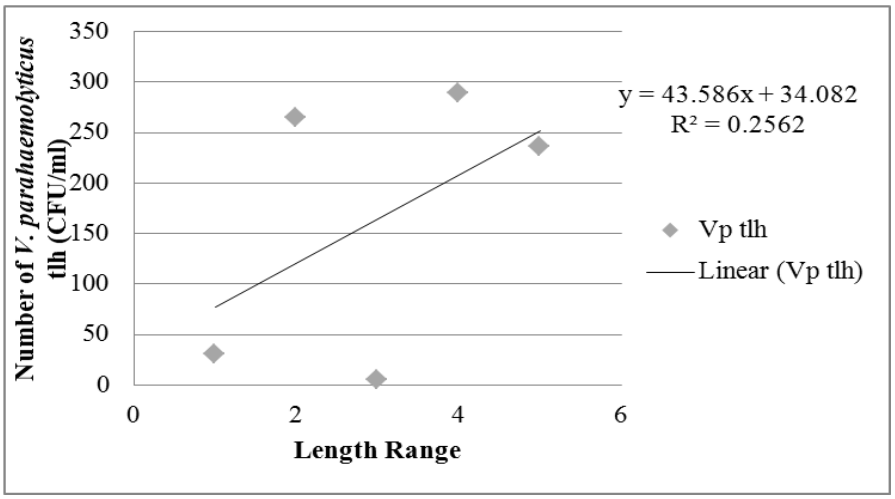


Figure II.6. Correlation of *V. parahaemolyticus tlh* abundance versus length of fish

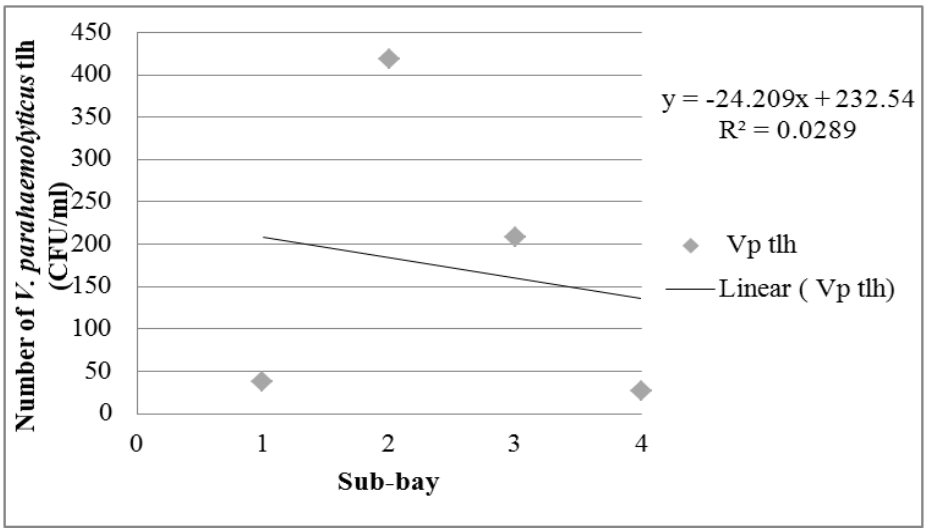


Figure II.7. Correlation of *V. parahaemolyticus tlh* abundance versus location of fish.

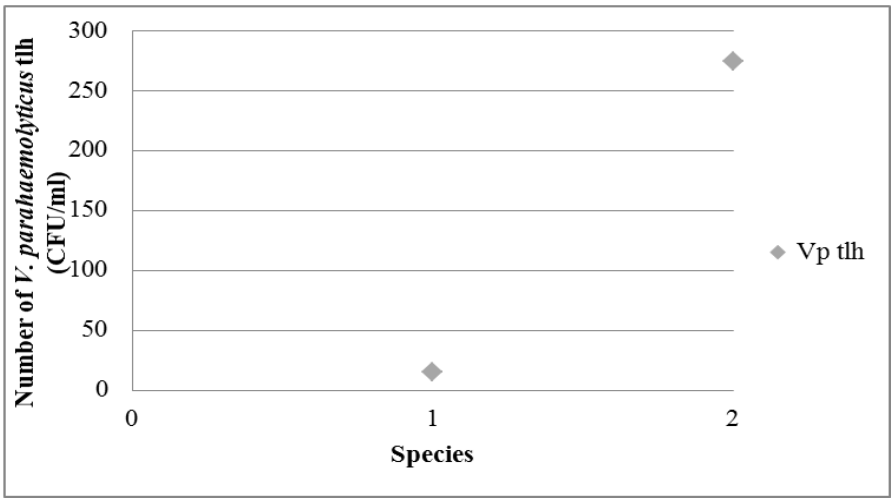


Figure II.8. Correlation of *V. parahaemolyticus tlh* abundance versus species of fish.

Table II.10. Kolmogorov-Smirnov results for *V. parahaemolyticus tlh*

	V. parahaemolyticus tlh
Kolmogorov-Smirnov Z	3.593
Asymp. Sig. (2 tailed)	0.000

A p value of less than 0.05 indicated that the data was normally distributed for the *V. parahaemolyticus tlh* data points (Table II.10.).

ANOVA was performed after normal distribution was determined to see if any relationship was significantly different between the number of bacteria found on the fish and length, location or species of the fish.

Post hoc tests were also run for if the ANOVA p value was less than 0.05, indicating a significant difference. These tests were Tukey's, for equal variance assumed, and Tamhane's T2, for unequal variance assumed. If there was a significant

difference, these tests would indicate which variable(s) was significantly different from the others.

Table II.11. ANOVA results for quantity of *V. parahaemolyticus tlh* versus length of fish.

	Sum of Squares	df	Mean Square	F	p
Between Groups	1016163.774	4	254040.943	.685	.605
Within Groups	2.707E7	73	370789.730		
Total	2.808E7	77			

The p value for the number of *V. vulnificus* versus length was 0.605. This is greater than a value of 0.05, which means that there is no difference in the number of bacteria based on the length of the fish (Table II.11.).

Table II.12. ANOVA results for quantity of *V. parahaemolyticus tlh* versus location of fish.

	Sum of Squares	df	Mean Square	F	p
Between Groups	788761.339	3	262920.446	0.713	0.547
Within Groups	2.730E7	74	368852.064		
Total	2.808E7	77			

The p value for the quantity of *V. parahaemolyticus tlh* versus the location of the fish was 0.547. This value is greater than 0.05, which indicates no significant difference in the quantity of bacteria based on the location of the fish (Table II.12.).

Table II.13. ANOVA results for quantity of *V. parahaemolyticus tlh* versus species of fish.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1210170.970	1	1210170.970	3.422	0.068
Within Groups	2.687E7	76	353600.567		
Total	2.808E7	77			

The p value for the number of *V. parahaemolyticus tlh* versus the species of fish was 0.068. This value was greater than 0.05, which shows no significant difference the number of bacteria based on the species of fish (Table II.13.). Because *V. parahaemolyticus tdh* did not yield any positive results, no linear regressions or statistical analysis could be done.

Vibrio cholerae

A total of 250 samples were also checked for *V. cholerae* presence. Of the samples tested for *Vibrio cholerae*, 23 or 9.2%, yielded positive results from the QPCR assay. Initial tests to check for the presence of *V. cholerae* was done by conventional PCR that targeted the *ctxAB* complex. Approximately 50 samples that had yielded

negative results were checked with QPCR. All confirmed the initial negative result and it was assumed that all other initially *Vibrio cholerae* negative samples would also produce a negative QPCR result.

The quantity of *V. cholerae* was much lower than for *V. vulnificus* and *V. parahaemolyticus*, ranging from 0 to 21 CFU/ml. The highest quantities were associated with fishes 20-21.5 centimeters total length. Unlike *V. vulnificus* and *V. parahaemolyticus*, *V. cholerae* were mostly associated with fishes in East Bay, followed by Galveston Bay, then Trinity Bay. Like *V. vulnificus* and *V. parahaemolyticus*, *V. cholerae* was predominantly associated with hardhead catfish (fig. II.9. A, B, C).

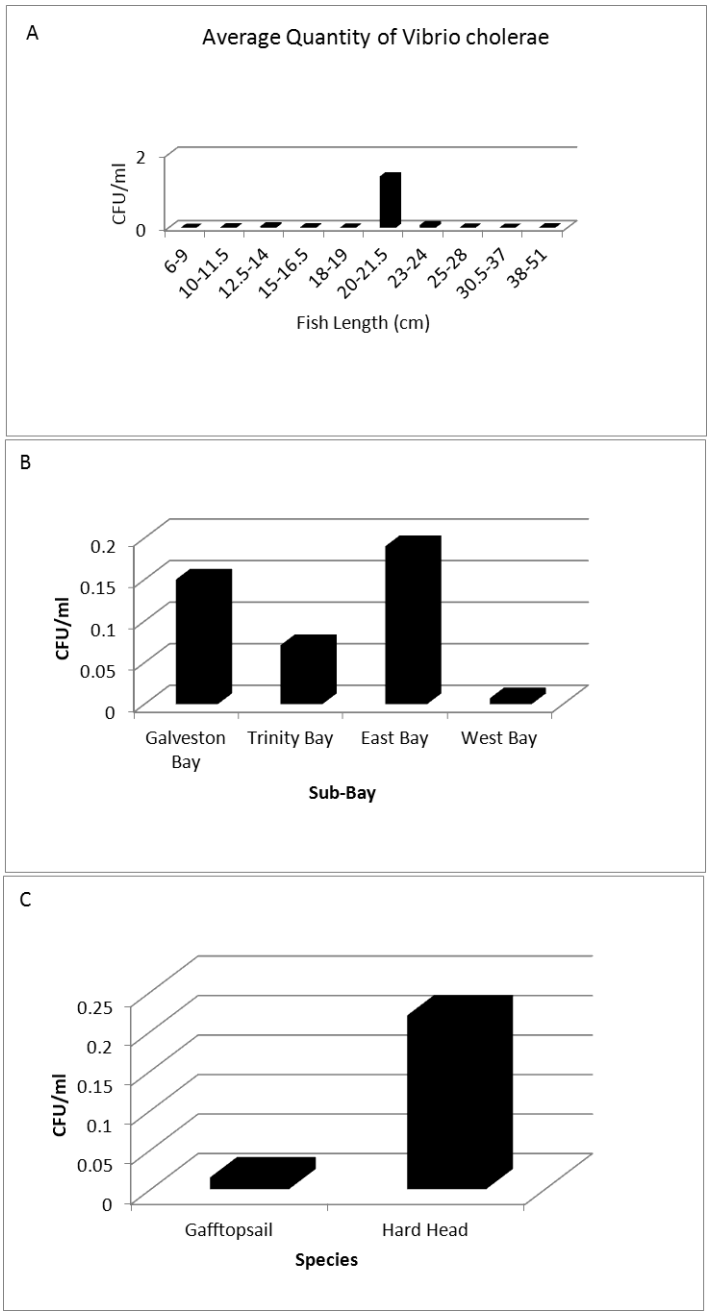


Figure II.9. Distribution of *V. cholerae* by A) fish length, B) sub-bay, and C) species of catfish

As with the other bacteria, linear regressions were run for *Vibrio cholerae* against length, location and species of fish. Group designations remain the same as with the other bacteria and can be found in table II.3.

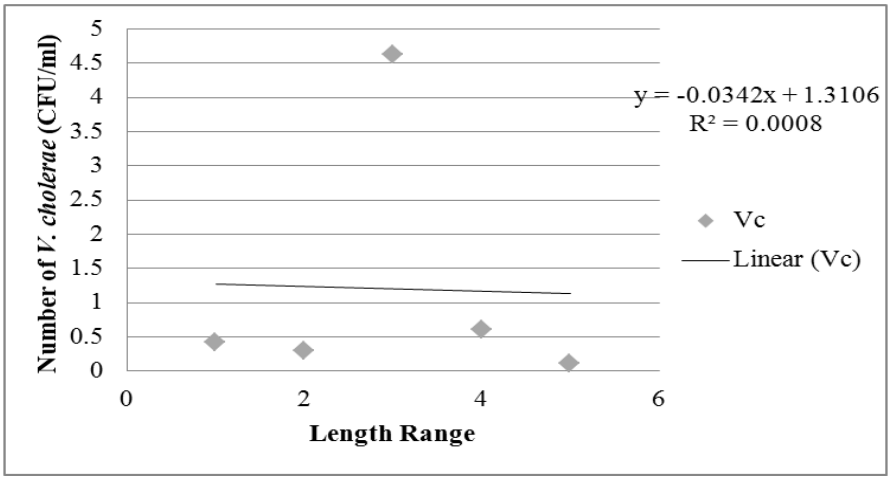


Figure II.10. Correlation of *V. cholerae* abundance versus length of fish.

An R^2 value of 0.0008 shows that there is virtually no relationship between the number of *V. cholerae* and the length of the fish (fig. II.10.).

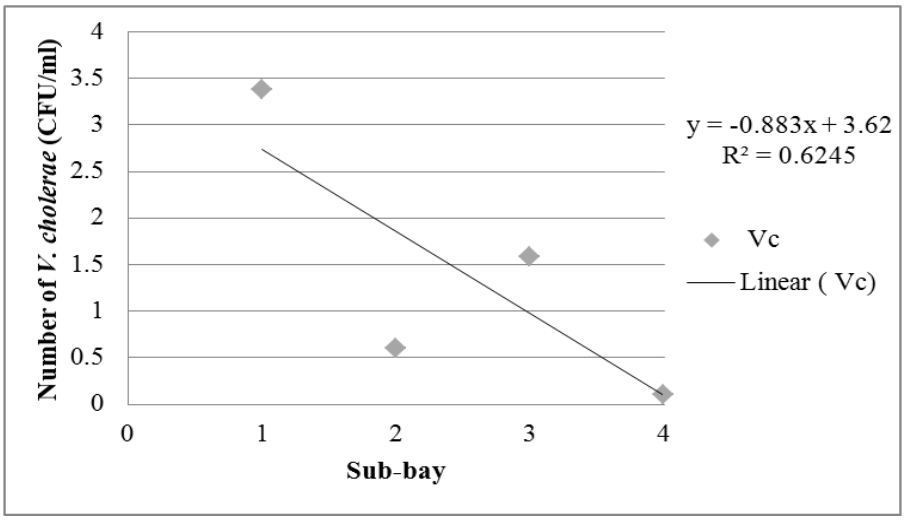


Figure II.11. Correlation of *Vibrio cholerae* abundance versus location of fish.

An R^2 value of 0.6245 shows a moderate relationship between the number of bacteria and the location of the fish (fig. II.11.).

Figure II.12. Correlation of *Vibrio cholerae* abundance versus species of fish.

A linear regression trendline was not added to figure II.12. as there were only two data points for comparison.

As with the other bacteria, more sensitive tests were done using SPSS to see if any of these relationships were significant. First, normal distribution of data was determined through a Kolmogorov-Smirnov test.

Table II.14. Kolmogorov-Smirnov results for *V. cholerae*

	V. cholerae
Kolmogorov-Smirnov Z	2.152
Asymp. Sig. (2 tailed)	0.000

A p value of less than 0.05 indicated that the data was normally distributed for the *V. cholerae* data points (table II.14.).

ANOVA was performed after normal distribution was determined to see if any relationship was significantly different between the number of bacteria found on the fish and length, location or species of the fish.

Post hoc tests were also run for if the ANOVA p value was less than 0.05, indicating a significant difference. These tests were Tukey's, for equal variance assumed, and Tamhane's T2, for unequal variance assumed. If there was a significant difference, these tests would indicate which variable(s) was significantly different from the others.

Table II.15. ANOVA results for quantity of *V. cholerae* versus length of fish.

	Sum of Squares	df	Mean Square	F	p
Between Groups	89.739	4	22.435	0.900	0.486
Within Groups	423.732	17	24.925		
Total	513.472	21			

The p value for the number of *Vibrio cholerae* versus length was 0.486. This is greater than a value of 0.05, which means that there is no difference in the number of bacteria based on the length of the fish (table II.15.).

Table II.16. ANOVA results for number of *V.cholerae* versus location of fish.

	Sum of Squares	df	Mean Square	F	p
Between Groups	15.874	3	5.291	0.201	0.894
Within Groups	500.267	19	26.330		
Total	516.141	22			

The p value for the quantity of *Vibrio cholerae* versus the location of the fish was 0.894. This value is greater than 0.05, which indicates no significant difference in the number of bacteria based on the location of the fish (table II.16.).

Table II.17. ANOVA results for quantity of *Vibrio cholerae* versus species of fish.

	Sum of Squares	df	Mean Square	F	p
Between Groups	12.206	1	12.206	0.487	0.493
Within Groups	501.266	20	25.063		
Total	513.472	21			

The p value for the number of *Vibrio cholerae* versus the species of fish was 0.493. This value was greater than 0.05, which shows no significant difference the number of bacteria based on the species of fish (table II.17.).

Discussion

This chapter tested both hypotheses:

Hypothesis 1: Benthic-dwelling fishes, such as *A. felis* and *B. marinus*, are important vectors for virulent *Vibrio vulnificus* and *Vibrio parahaemolyticus*.

Hypothesis 2: Quantitative Polymerase Chain Reaction and will be better assays for determining presence or absence of *Vibrio vulnificus* and *Vibrio parahaemolyticus* than more conventional microbial methods.

Catfish as a Vector

Ariopsis felis and *B. marinus* tend to favor environments that are also favorable to *Vibrio* spp. bacteria. With *A. felis* and *B. marinus* being benthic dwellers, it is likely that they acquired these bacteria while swimming or foraging for food. During

unfavorable times, it is possible that these *Vibrio* spp. bacteria are using the catfish as a means of finding a more favorable environment. The quantities of *V. vulnificus* and *V. parahaemoltyicus* were below the infectious dosage of 10^6 for ingestion, but this dosage may be lower for direct stab wound to soft tissues.

Comparison of QPCR and Gene Probe

As a method of detection, QPCR proved to be more sensitive when detecting the bacteria in samples. Samples that had initially been confirmed as negative for bacteria using Gene Probe were discovered to be positive for bacterial DNA when run through QPCR. Since QPCR looks at DNA, it can be determined that the DNA came from one of two sources. The first potential source of the DNA could have been from dead bacteria present on the fin. The other source could be from bacteria that had entered into a VBNC state. In order to determine which source it was, microscopy would have to be conducted to look at the cell morphology.

However, there were many samples that had initially yielded positive results via Gene Probe that did not yield results with the QPCR. Most likely, this can be attributed to a lag in time from the initial study using Gene Probe, and when the QPCR assay was done. If possible, for future studies, the two assays should be done on a smaller time scale. This will most likely improve not only the number of positive results, but also the number of bacteria found per sample.

Statistical Analysis

For statistical analysis, only the samples that yielded results were used. Those samples that peaked with QPCR, but at too late a cycle to be detected were assigned a value of 0.1 CFU/ml. This number was determined based on the lowest number of CFUs that were detected for other QPCR samples. For *V. vulnificus*, there was a statistical outlier with a value of 52,565.94 CFU/ml. This data point was excluded from analysis since it was three orders of magnitude larger than the next closest data point and skewed the statistical analysis.

Most of the ANOVAs that were run showed that there was no significant difference between the number of bacteria found on the fish in relation to our set parameters. The relationship between the abundance of *V. parahaeomolyticus tlh* and species yielded a p value of 0.068 for the ANOVA. Given this, it is possible that *V. parahaemolyticus* have a preference for one species over another. It is possible, that if the assay had been done sooner after collection had occurred that a significant difference might have been detected.

CHAPTER III

ANALYSIS OF *VIBRIO* SPP. BACTERIA ON HARDHEAD AND GAFFTOPSAIL CATFISH USING TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP)

Introduction

In Chapter II, it was determined that *Ariopsis felis* and *Bagre marinus* are an important reservoir, and potentially an important vector for human infection, of *V. vulnificus* and *V. parahaemolyticus*. A previous study of the population structure of *V. vulnificus* in Galveston Bay found two strains, A and B, that can be differentiated in the 16S ribosomal RNA, exhibited seasonal variation in their relative abundance (Lin and Schwarz, 2003). The diversity of *Vibrio* bacteria, in particular strains or biotypes of *V. vulnificus* and *V. parahaemolyticus*, may provide additional information about the importance of catfish as reservoirs for the bacteria. There is also increasing evidence that clinical strains of these bacteria are more virulent than those isolated after traditional enrichments with Alkaline Peptone Water (APW). The catfish skin and slime may provide the elements missing from APW that could select for the more virulent strains. In this chapter, T-RFLP, a rapid method for bacterial community DNA fingerprinting, was used to determine if multiple species of *Vibrio* bacteria are associated with *A. felis* and *B. marinus.*

Materials and Methods

Sample Collection

In the summer of 2006, hardhead (*A. felis*) and gafftopsail (*B. marinus*) catfish were collected with the assistance of the Texas Parks and Wildlife Dickinson Marine Lab in conjunction with their fish population studies and local Galveston shrimpers. Fish were also collected in the fall of 2006 through the Texas Parks and Wildlife Dickinson Marine Lab gill net program. Fish were transported to the lab on ice where spines and fins were removed for bacterial culturing.

Processing of Samples

Fins and spines were removed using sterile forceps and bone cutting pliers. The dorsal fin together with its spine and one of the pectoral fins together with its spine from each individual fish were transferred to 15-ml conical tubes containing 5 ml of alkaline peptone water (APW) for 24 h cultivation at 35 °C. All, or part, of the second pectoral fin and spine from each fish was transferred to 2-ml tubes and frozen at -20 °C for later DNA extraction. The cultivated *V. vulnificus* and *V. parahaemolyticus* bacteria were isolated using media specific for each species and then further analyzed for toxicity genes, i.e. vvh and *tdh*/*tlh* respectively, using alkaline phosphatase labeled DNA probes as described Wright et al. (1993) and Drake et al. (2007). Bacteria were grown on selective media for isolation over a period of three days. Isolated colonies were then affixed to Whatman filters via microwave. Filters were then put through a series of washes for neutralization and to remove any background alkaline phosphatase activity.

After being washed, the filters were then hybridized with alkaline phosphatase probes and chemically visualized with NBT-BCIP.

Extraction of Nucleic Acids

Bacterial DNA from pectoral fins stored at -20 °C was extracted using a Cetyltrimethyl Ammonium Bromide (CTAB)/chloroform-isoamyl alcohol method (Doyle and Doyle, 1990). In short, fins were incubated in a 3% CTAB buffer (500 µl) at 65°C for 30 min. Chloroform-isoamyl alcohol (500 µl) was added to the sample, vortexed, and then centrifuged to separate the DNA from the cell material. The aqueous top layer was transferred to a clean tube and 100% cold isopropyl alcohol (60% volume) was added to precipitate the DNA. DNA was harvested by centrifugation, supernatant discarded, pellet washed with cold 80% ethanol and air dried. DNA was re-suspended in 200 µl low Tris (LT) buffer (dilute Tris-Acetate-EDTA, TAE, buffer), analyzed for concentration and purity with a Nanodrop spectrophotometer, and stored at -20 °C for later processing.

Terminal Restriction Fragment Length Polymorphism

Terminal Restriction Fragment Length Polymorphism (T-RFLP) is one of the most commonly used high-throughput techniques for determining community structure (Pandey et al., 2007). According to Kim and Marsh (2008), it is a variant of conventional RFLP analysis where the fragments derived from the digestion of a PCR-amplified target are electrophoretically sized, a technique also know as Amplified Ribosomal DNA

Restriction Analysis (ARDRA). The only difference between T-RFLP and ARDRA is that in T-RFLP only the fragment closest to a fluorescently labeled primer is detected and sized on an automated gel or capillary system (Kim and Marsh, 2008).

Extracted DNA was amplified using two different primers targeting the 16s rRNA: 27F, which is a bacteria specific primer, and 680R, which is a *Vibrio* spp. specific primer (Thompson et al. 2004). The sequences for both can be found in table III.1. The forward primer was labeled with the fluorescent dye 6' FAM at the 5' end. The fluorescent dye 6' FAM was chosen based on its ability to identify the most number of species and ribotypes as demonstrated in Pandey et al. (2007).

Table III.1. T-RFLP primers.

Primer Name	Sequence	Reference
27F	AGAGTTTGATCMTGGCTCAG	Tompson et al., 2004
680R	GAATTCTACCCCCCTCTACAG	

Samples were run through PCR first using the following protocol:

Initial Denaturation	95°C	3 min
Denaturing	94°C	45 sec
Annealing	57°C	1 min
Elongation	72°C	2 min
Repeated 30 times		
Final Elongation	72°C	7 min

Product from the PCR was then run through Speedvac for approximately 1 h to dry samples. Dried samples were then rehydrated with 10 µl of PCR water before being loaded into a 2% agarose gel. Gels were run for approximately 90 min at 70 volts. Gels were removed from the system in complete darkness and placed into a SYBR-Gold staining bath for 30 min. Afterwards, gels were quickly documented under UV light using a gel documentation system (BioRad, Hercules, CA), and then immediately transferred to a blue light transilluminator (Clare Chemical Research, Dolores, Co). Blue light (400 - 500 nm) does not nick DNA and can be used for longer analysis times.

An ethanol-sterilized razor blade was used to excise single bands from the gel and stored in 1.5-ml Eppendorf tubes at -20°C. Gel slices were then put into 50 ul of PCR-grade water overnight to elute DNA. Before proceeding on, concentrations were checked with Nanodrop to make sure that there was sufficient amount of product.

Next, a restriction digest was done on samples using a 4 base pair cutter. The restriction enzyme HaeIII was used for this study. HaeIII is a high-frequency cutting restriction enzyme that has a high average number of restriction sites and comes from the organism, *Haemophilius aegyptius*. The recognition sequence for this enzyme is:

HaeIII 5’GG^CC3’

Digests were incubated at 40°C overnight to allow for complete digestion. Once the digests were complete, samples were desalted by adding 600 µl of 95% ethanol to the sample and placing it in the freezer for 3 h. Samples were then centrifuged at 18894 x g for 10 minutes at 4°C. After centrifuging, the ethanol was decanted and samples were allowed to dry. Dried samples were then resuspended in 20µl of LT buffer.

Prior to running on the sequencer, 4 µL of each sample was first cleaned with 1 µL of ‘Exosap it’ and run on the thermal cycler using the following protocol:

80 °C for 15 minutes

35 °C for 15 minutes

Once the samples were clean, 0.5 µL of Rox 2500 standard was fixed using 22 µL of formamide. These were then run for another 3 minutes at 95 °C on the thermal cycler. At this point, samples were then loaded onto 96-well plates to be run on the sequencer.

The samples were run on the ABI 3130 Sequencer (Applied Biosystems, Carlsbad, CA). Default protocols for the AFLP program were used with the standard injection time changed from 10 s, to 60 s to maximize peak detection.

Samples were analyzed using Gene Mapper software (Applied Biosystems, Carlsbad, CA). A light smoothing was applied to the baseline to lower background noise. Allele labels and thresholds were set to the values listed in table III.2.

Table III.2. Allele labels and threshold levels

Threshold	Allele Label
30	0
50	check
	1

Only a partial range was looked at, from 40 bp to 500 bp in length as the total PCR product length was 653 bp.

Results

T-RFLP worked well as a rapid screening method for diversity of *Vibrio* bacteria. A total of 245 samples were analyzing using T-RFLP. Of those samples, 172 or 70%, produced at least one peak. Table III.3. shows the comparison of gene probe to T-RFLP analysis.

Table III.3. Comparison of gene probe analysis and T-RFLP analysis

Gene Probe Positive			Gene Probe Negative		
T-RFLP Positive	T-RFLP Negative	Total	T-RFLP Positive	T-RFLP Negative	Total
168	66	234	4	7	11

Of those samples that yielded a positive result with gene probe (whether one bacteria or all three), 168 or approximately 72% were also positive using T-RFLP. Of those samples that yielded negative results, 4 or 36%, gave a positive result with T-RFLP. One of those four samples did test positive for *V. cholerae* with conventional PCR.

Standards were run first to determine the number of peaks and size for the bacteria sampled. Both *V. vulnificus* and *V. parahaemolyticus* produced two bands, one that was approximately 130 base pairs in size, and one that was approximately 205 base pairs in size. For *V. cholerae,* only one band was produced around 205 base pairs in size.

T-RFLP analysis revealed 28 different sequence fragments of *Vibrio* spp. bacteria associated with catfish samples. Fragment lengths ranged from 51 base pairs in

length, to 219 base pairs in length. These fragments were then grouped into ranges based on sizes. Ranges are listed in table III.4.

Table III.4. List of fragments produced by T-RFLP and their frequency.

Allele	Size (bp)	Frequency
1	51 - 64	14
2	117-121	22
3	143-150	58
4	158-186	3
5	213-219	163

Each fragment, or allele, produced corresponds to the band containing the 27F phosphorescent labeled primer. Peaks were produced that correspond to the different bands found in each sample. Figure III.1. shows a chromatograph that was produced during the analysis.

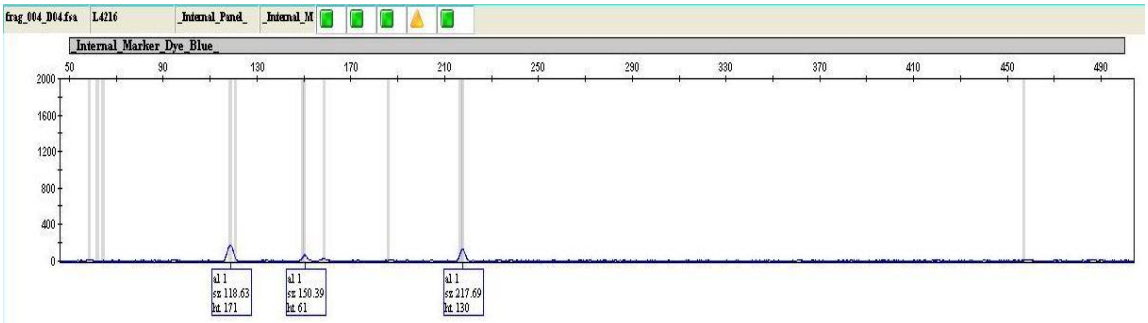


Figure III. 1. Chromatograph of environmental sample from East Bay.

None of the sample peaks that were produced were an exact match to those peaks found by the standard, but many fell within about 20 base pairs of the base pair sizes for the standards that were included for comparison. In particular, these alleles are 3 and 5. This difference in size may be attributed to multiple biotype or strains and could be confirmed with sequencing. The most frequently occurring allele was number 5, with 163 samples containing it. The rarest allele was allele 4, which was only found in 3 samples.

The number of peaks per sample ranged from zero to four, with the average being one peak per sample. A breakdown of percentages of number of peaks can be found in Figure III.2. A complete table of samples and alleles can be found in Appendix A.

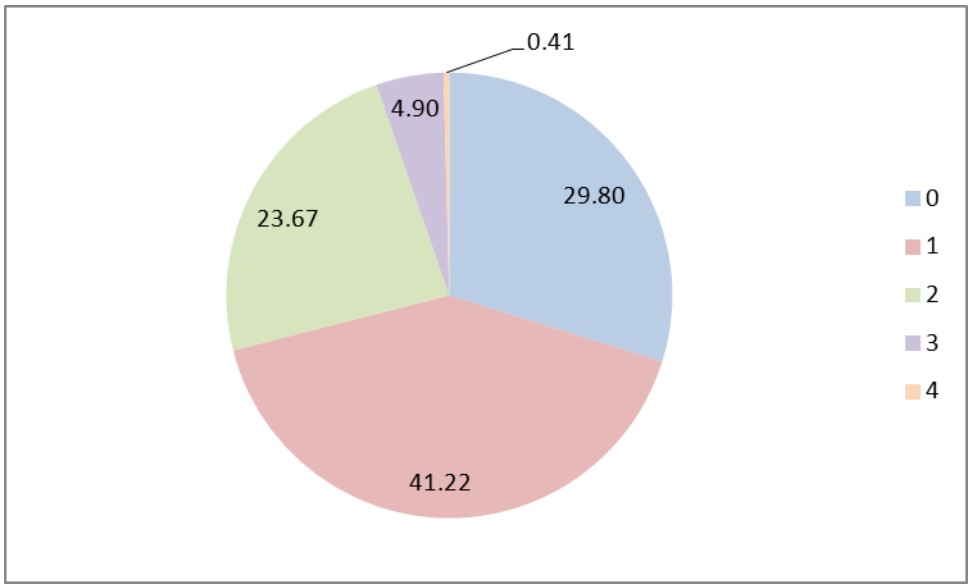


Figure III.2. Percentage of number of peaks detected using T-RFLP.

Nearly half the samples contained one peak. Those that produced no peaks were the next frequent, followed closely by samples that produced two peaks. Of those that produced two peaks or more peaks, 57 samples, or 80%, contained both alleles 3 and 5.

Like with QPCR, allele frequency was compared to three different parameters: length, location and species of fish. These frequencies can be found in tables III.5-7.

Table III.5. Frequency of alleles compared to length of fish.

Length Range	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5
6-12.5 cm	2	2	10	0	20
12.5-18 cm	3	10	20	1	65
18-23 cm	3	4	15	0	35
23-28 cm	2	5	5	1	15
28-51 cm	4	1	8	1	28

The 12-18 cm length range had the highest frequencies for all the alleles except for the first and fourth allele.

Table III.6. Frequency of alleles compared to location of fish.

Location	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5
Galveston Bay	3	3	18	0	42
Trinity Bay	2	1	4	0	11
East Bay	8	18	31	3	85
West Bay	1	0	5	0	23

For the location, East Bay had the highest frequencies for all alleles.

Table III.7. Frequency of alleles compared to species of fish.

Species	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5
Hard head	11	15	36	2	101
Gafftopsail	3	7	22	1	62

Hardhead catfish had the higher frequencies for all alleles.

Discussion

This chapter tested the second hypothesis:

Hypothesis 2: Terminal Restriction Fragment Length Polymorphism will be better assays for determining presence or absence of *Vibrio vulnificus* and *Vibrio parahaemolyticus* than more conventional microbial methods.

Comparison of Gene Probe and T-RFLP

As a method of determining presence and absence, T-RFLP worked very well. It had a high rate of success with 70% of samples yielding results. However, T-RFLP can be a very time intensive process. T-RFLP is also not sensitive enough to determine the bacteria to the species. Further processing of the samples would be needed in order for the species to be determined.

Frequency of Alleles

Based on the sizes of the standards, it can be determined that any samples that contained both alleles 3 and 5 were most likely positive for *V. vulnificus,*, *V. parahaemolyticus* or both. Samples that contained allele 5, either alone or in combination with other alleles, most likely were positive for *V. cholerae*. Other alleles and allele combinations are most likely other species of *Vibrio* that are present on the fish fins. Further analysis of these sequences would be needed to confirm each bacterial species.

Looking at the test parameters, some trends were observed. For length, the favored range was the 12-18 cm range. This group had the highest frequencies for all but the first and fourth allele. Having the highest frequencies, these fish probably have the most diversity of *Vibrio* spp. bacteria. East Bay had the highest frequencies for all alleles, which suggests that East Bay contains the most diversity of all the bays sampled. For species, there was an overwhelming favoring of hardhead catfish with allele frequencies, and consequently that species had a higher diversity of bacteria.

As a screening tool, T-RFLP can be used to identify samples requiring further characterization. In this study, T-RFLP provided an important first step in examining the diversity of *Vibrio* bacteria associated with catfish in Galveston Bay.

CHAPTER IV

CONCLUSION

Conclusions

Vibrio *spp. associated with catfish*

Previous studies on *Vibrio* spp. bacteria have studied them in or on several mediums: water (Blackwell et al., 2008; Lin and Schwarz, 2003), soil (Blackwell et al., 2008), oysters (Novotny et al., 2004; Paniker and Bej, 2004; Lin and Schwarz, 2003) and fish guts (DePaola et al., 1994). This, however, is the first study to really look at the presence of these bacteria on an external surface of a fish. Overall, this thesis showed that these bacteria are present on the majority of fins and spines of *A. felis* and *B. marinus.*

Factors that determine the relationship of these *Vibrio* spp. bacteria on catfish are not yet known. Future research should be conducted to characterize the outer slime layer of catfish for nutrients, i.e. proteins or polysaccharides, that make catfish a suitable habitat for the *Vibrio* bacteria, and whether the catfish just serve as a means of getting the bacteria from unfavorable habitats to more favorable habitats. The latter would be more likely during colder months when temperatures begin to drop and bacteria shift to a VBNC state. Further research on community structure may help with tracking the source of these bacteria on the fish.

This study focused mainly on two of the *Vibrio* spp. bacteria that are highly pathogenic to humans. These bacteria were chosen for the threat of infection to humans,

in particular fishermen, when they come into contact with these fishes. Based just on presence of the bacteria on the catfish fins, it can be determined that *A. felis* and *B. marinus* are a potential vector for human infection through puncture wounds. Especially during summer months, when levels of *Vibrio* spp. are higher, information about infections should be made available to those who are susceptible to infection. Doctors and hospitals can also benefit from this information. If the medical community knows when infections are more likely to occur, they can be ready to treat infections and reduce the risk of limb loss or even death. And, in turn, better documentation of the type of organism that caused the wound infection would provide much needed information about the risks of exposure.

Methods of Detection

For this thesis, three different methods were used and compared for detecting *Vibrio* spp. bacteria. The first method, gene probe, is a microbial method that has been used for years to determine the presence and absence of *Vibrio* spp. bacteria. While this is generally a fairly accurate method, it can be time consuming. Gene probe is limited in that it can only detect bacteria that are culturable, and therefore excludes bacteria that were dead or in a VBNC state.

The second method utilized was QPCR. This method proved to be the fastest of the three methods tested. Since QPCR tests DNA, rather than culturable cells, it can also detect things that gene probe cannot. If there were dead bacteria or cells in a VBNC state present on the fins, their DNA would get extracted and would be consequently detected

with QPCR. Overall, this was the best method for rapid detection of *Vibrio* spp. bacteria. This method would be extremely useful for clinical testing. Confirmation of *Vibrio* infection could be done in a fraction of the time it would take with microbial methods.

The third method utilized was T-RFLP. Much like QPCR, this method detects DNA and can sense both culturable and non-culturable cells in a sample. T-RFLP, like gene probe, can be a time consuming process. Also, T-RFLP is not sensitive enough to detect the exact species, which both gene probe and QPCR can do. In order to determine species, the bands would need to be sequenced and compared to sequences in a database like GenBank.

Future studies include determining if *V. vulnificus* and *V. parahaemolyticus* that occur on catfish represent 'clinical' strains that have higher virulence than those isolated from water, oysters, and surfaces in the marine environment. Catfish should be collected in winter months to determine if there is a seasonal shift in the biotype of *V. vulnificus* observed by Lin and Schwarz (2003) in Galveston Bay. And finally, screening of additional species of finfishes and other marine organisms for presence of toxigenic *Vibrio* bacteria should be conducted.

REFERENCES

Baack, B.R., J.O. Kucan, E.G. Zook and R.C. Russell. 1991. Hand infections secondary to catfish spines: case reports and literature review. Journal of Trauma. **31**: 1432-1436.

Baffone, W., B. Citterio, E. Vittoria, A. Casaroli, R. Campana, L. Falzano and G. Donelli. 2003. Retention of virulence in viable but non-culturable halophilic *Vibrio* spp. International Journal of Food Microbiology. **89**: 31-39.

Beckman EN, Leonard GL, Castillo LE, Genre CF, Pankey GA. Histopathology of marine *Vibrio* wound infections. Am J Clin Pathol 1981; 76: 765–772.

Blackstone, G.M., J.L. Nordstrom, M.D. Bowden, R.F. Meyer, P. Imbro and A. DePaola. 2006. Use of a real time PCR assay for detection of *ctx*A gene of *Vibrio cholerae* in an environmental survey of Mobile Bay. Journal of Microbiological Methods. **68**: 254-259.

Blackwell, K.D. and J.D. Oliver 2008. The ecology of *Vibrio vulnificus, Vibrio cholerae,* and *Vibrio parahaemolyticus* in North Carolina estuaries. The Journal of Microbiology. **46**: 146-153.

Bonner, J.R., A.S. Coker, C.R. Berryman and H.M. Pollock. 1983. Spectrum of *Vibrio* infections in a Gulf Coast community. Annals of Internal Medicine. **99**: 464-469.

Borenstein, M. and F. Kerdel. 2003. Infections with *Vibrio vulnificus.* Dermatol Clin. **21**: 245-248.

Brinkmeyer, R., L.D. Gilbert, M. Hochman and J.R. Schwarz. Association of toxigenic *Vibrio vulnificus, Vibrio parahaemolyticus* and *Vibrio cholerae* with hardhead and gafftopsail catfish in Galveston Bay: implications for human infection. In preparation.

Calif, E., B. Kaufman and S. Stahl. 2003. *Vibrio vulnificus* infections of the lower limb after fish spine injuries. Clinical Orthopaedics and Related Research. **411**: 274-279.

Chang-Chien, C., H. Ding, C. Lui and C. Yang. 2007. *Vibrio* infections associated with finning injury of the hand. Injury, Int. J. Care Injured. **38**: 614-618.

Colmer-Hamood, J.A. 2007. *Vibrio* in the United States: *Vibrio cholerae* and other pathogenic Vibrios. Lab Medicine. **38**: 688-692.

Daniels, N.A., L. MacKinnon, R. Bishop, S. Altekruse, B. Ray, R.M. Hommond, S. Thompson, S. Wilson, N.H. Bean, P.M. Griffin and L. Slutsker. 2000. *Vibrio*

parahaemolyticus infections in the United States, 1973-1998. The Journal of Infectious Disease. **181**: 1661-6.

Dechet, A.M., P.A. Yu, N. Koram and J. Painter. 2008. Nonfoodborne *Vibrio* infections: an important cause of morbidity and mortality in the United States, 1997-2006. Clinical Infectious Diseases. **46**: 970-976.

DePaola, A., G.M. Capers and D Alexander. 1994. Densities of *Vibrio vulnificus* in the intestines of fish from the U.S. Gulf Coast. Applied and Environmental Microbiology. **60**: 984-988.

DePaola, A., Motes, M.L., Cook, D.W., Veazey, J., Garthright, W.E., and Blodgett, R. 1997. Evaluation of an alkaline phosphatase-labeled DNA probe enumeration of *Vibrio vulnificus* in Gulf coast oysters. Journal of Microbiology Methods. 29(2):115-120.

DePaola, A., Nordstrom, J.L., Bowers, J.C., Wells, G., and D.W. Cook. 2003. Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. Applied Environmental Microbiology. 69:1521-1526.

Doyle, J. J. and J. L. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus. **12**: 13 - 15.

Drake, S.L., A. DePaola and L. Jaykus. 2007. An overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus.* Comprehensive Reviews in Food Science and Food Safety. **6**: 120-144.

Galveston Bay Estuary Program. 2010. Available: http://www.gbep.state.tx.us.

Galveston Bay Foundation. 2009. Available: http://www.GBF.org.

Heid, C.A., J. Stevens, K.J. Livak, and P.M. Williams. 1996. Real Time Quantitative PCR. Genome Res. **6**: 986-994.

Jiang, X.P. and T.J. Chai. 1996. Survival of *Vibrio parahaemolyticus* at low temperatures under starvation conditions and subsequent resuscitation of viable, nonculturable cells. Applied and Environmental Microbiology. **62**: 1300-1305.

Joseph, S.W., R.R. Colwell and J.B. Kaper. 1982. *Vibrio parahaemolyticus* and related halophilic Vibrios. CRC Critical Reviews in Microbiology. **10**: 77-124.

Kasper J., Lockman H., Colwell, R. R. Joseph, S.W. 1979. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. Applied Environmental Microbiology. **37**:91-103.

Klontz, K.C., S. Lieb, M. Schreiber, H.T. Janowski, L.M. Baldy and R.A. Gunn. 1988. Syndromes of *Vibrio vulnificus* infections: clinical and epidemiologic features in Florida ases, 1981-1987. Annals of Internal Medicine. **109**: 318-323.

Kim, S. and T.L. Marsh. 2008. The analysis of microbial communities with terminal restriction fragment length polymorphism (T-RFLP), *In* Kowalchuck, G.A., F.J. de Brujin, I.M. Head, A.D.L. Akkermans and J.D. van Elsas (2 ed), Molecular Microbial Ecology Manual. Springer. Dordrecht, The Netherlands. p. 3.15/789-808.

Lie, Y.S. and C.J. Petropoulos. 1998. Advances in quantitative PCR technology: 5' nuclease assays. Biotechnology. **9**: 43-48.

Lin, M, and J.R. Schwarz. 2003. Seasonal shifts in population structure of *Vibrio vulnificus* in an estuarine environment as revealed by partial 16S ribosomal sequencing. FEMS Microbiology Ecology. **45**: 23-27.

Midani, S. and M.H. Rathmore. 1994. *Vibrio* species infection of a catfish spine puncture wound. The Pediatric Infectious Disease Journal. **13**: 333-334.

Morris, J.G., Wilson, R., Davis, B.R., Wachsmuth, I.K., Riddle, FD.F., Wathen, H.G., Pollard, R. A., and Blake, P.A. 1981. Non-O group 1 *Vibrio cholerae* gastroenteritis in the United States. Annals of Internal Medicine. 94:656-658.

Muncy, R .J, and W.M. Wingo. 1983. Species profiles: life histories and environmental requirements of coastal invertebrates (Gulf of Mexico) – sea catfish and gafftopsail. U.S. Fish and Wildlife Service, Division of Biological Services, FWS/OBS-82/11.5. U.S. Army Corps of Engineers, TR EL-82-4. 17 pp.

Nishino, T. and K. Kogure. 2003. Transient temperature up-shift suppress the loss of culturability of *Vibrio parahaemolyticus* stressed by low temperature and nutrient deficiency. Microbes and Environments. **18**: 227-230.

Nordstrom, J.L., M.C.L. Vickery, G.M. Blackstone, S.L. Murray and A. DePaola. 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. Applied and Environmental Microbiology. **73**: 5840-5847.

Novotny, L., L. Dvorska, A. Lorencova, V. Beran and I. Pavlik. 2004. Fish: a potential source of bacterial pathogens for human beings. Veterinary Medicine – Czech. **49**: 343-358.

Ohio Department of Health. 2009. Vibriosis. Ohio Department of Health, Ohio. Available: http://www.odh.ohio.gov.

Oliver, J.D. 2005 The viable but nonculturable state in bacteria. The Journal of Microbiology. **43**: 93-100.

Pandey, J., K. Ganesan, and R.K. Jain. 2007. Variations in T-RFLP profiles with differing chemistries of fluorescent dyes used for labeling the PCR primers. Journal of Microbiological Methods. **68:** 633-638.

Paniker, G. and A.K. Bej. 2004. Real time PCR Detection of *Vibrio vulnificus* in Oysters: a comparison of oligonucleotide primers and probes targeting *vvhA.* Applied and Environmental Microbiology. **71**: 5702-5709.

Ralph, A. and B.J. Currie. 2007. *Vibrio vulnificus* and *V. parahaemolyticus* necrotising fasciitis in fishermen visiting an estuarine tropical northern Australian location. Journal of Infection. **54**: 111-114

Smith, B. and J.D. Oliver. 2006. In situ and in vitro gene expression by *Vibrio vulnificus* during entry into, persistence within, and resuscitation from the viable but nonculturable state. Applied and Environmental Microbiology. **72**: 1445-1451.

Smithsonian Maine Station at Fort Pierce. 2010. Field guide to Indian River Lagoon, Florida. Available: http://www.sms.si.edu/irlfieldguide/BonyFishes.htm

The Jump. 2010. Hardhead catfish. Available: http://www.thejump.net/id/hardhead-catfish.htm.

Thompson, J.R., M.A. Randa, L.A. Marcelino, A. Tomita-Mitchell, E. Lim and M.F. Polz. 2004. Diversity and dynamics of a North Atlantic Coastal *Vibrio* community. Applied and Environmental Microbiology. **70**: 4103-4110

Tsai, Y., R.W. Hsu, K. Huang, C. Chen, C. Cheng, K. Peng and T. Huang. 2004. Systemic *Vibrio* infection presenting as necrotizing fasciitis and sepsis. The Journal of Bone and Joint Surgery. **86-A**: 2497-2502.

Wolf, P.W. and J.D. Oliver. 1992. Temperature effects on the viable but non-culturable state of *Vibrio vulnificus*. FEMS Microbiology Ecology. **101**: 33-39.

Wright, A.C., G.A. Miceli, W.L. Landry, J.B. Christy, W.D. Watkins and J.G. Morris Jr. 1993. Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphotase-labeled oligonucleotide probe. Applied and Environmental Microbiology. **59**: 541-546.

APPENDIX A

Table of alleles for T-RFLP

Sample Name	Sample Number	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Number of Peaks
L421	60						0
L422	61		+				1
L423	62						0
L424	63	+					1
L425	66		+			+	2
L426	67		+		+	+	3
L427	68		+			+	2
L429	70	+	+			+	3
L4210	71		+				1
L4211	72		+			+	2
L4213	74		+				1
L4214	76	+	+				2
L4215	82	+	+			+	3
L4217	85	+	+			+	3
L4218	86		+		+		2
L4219	87					+	1
L4221	90		+			+	2
L4222	91	+	+				2
L4223	92		+			+	2
L4225	95						0
L4226	96						0
L4227	97						0
L4229	99						0
L4230	100						0
L4231	101						0
L4232	102					+	1
L4233	103						0
L4234	104						0

Sample Name	Sample Number	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Number of Peaks
L4235	105	+					1
L4237	107						0
L4238	108	+					1
L4239	109						0
L4241	111						0
L4242	112						0
L4245	115					+	1
L4246	116						0
L4247	117						0
L4249	119						0
L4250	120						0
L4251	121						0
L4253	123					+	1
L4254	124						0
L4255	125					+	1
L4257	128						0
L4258	129						0
L4259	130						0
L4261	132						0
L4262	133						0
L4263	134						0
L4265	136					+	1
L4266	137					+	1
L4267	139						0
L4269	141					+	1
L4270	142						0
L4283	159					+	1
L4285	161					+	1
L4286	162					+	1
L433	166					+	1
L434	167						0
L435	168					+	1
L437	170					+	1
L439	172						0
L4310	173					+	1

Sample Name	Sample Number	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Number of Peaks
L4312	175					+	1
L4313	176						0
L4314	177					+	1
L4316	179					+	1
L4317	180						0
L4320	182					+	1
L4321	184						0
L4322	185					+	1
L4324	187					+	1
L4326	189					+	1
L4328	191					+	1
L4329	194						0
L4332	200					+	1
L4333	202					+	1
L4334	203					+	1
L4336	206					+	1
L4337	208					+	1
L4339	211					+	1
L4342	214						0
L4343	215					+	1
L4345	217						0
L4346	218					+	1
L4347	219					+	1
L4349	222					+	1
L4350	223					+	1
L4351	225					+	1
L4353	227					+	1
L4354	228					+	1
L4355	229					+	1
L4357	230						0
L4358	231						0
L4359	232					+	1
L4361	234						0
L4362	236						0
L4363	237					+	1
L4365	239					+	1
L4366	240						0

Sample Name	Sample Number	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Number of Peaks
L4385	272					+	1
L4386	273						0
L4387	274					+	1
L4388	276					+	1
L4389	277						0
L4390	278						0
L4391	279					+	1
L441	280					+	1
L445	284						0
L446	285		+			+	2
L447	286					+	1
L449	289						0
L4410	290						0
L4411	291					+	1
L4413	294						0
L4414	295					+	1
L4415	296					+	1
L4417	298						0
L4418	299						0
L4419	300					+	1
L4421	302						0
L4422	303						0
L4425	306						0
L4426	307						0
L4427	308					+	1
L4429	310						0
L4430	311						0
L4431	312					+	1
L4433	314					+	1
L4434	315	+					1
L4435	317					+	1
L4437	320					+	1
L4438	322						0
L4439	323					+	1
L4440	324					+	1
L4441	325						0
L4442	326						0

Sample Name	Sample Number	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Number of Peaks
L4443	328					+	1
L4445	330					+	1
L4446	333					+	1
L4447	334					+	1
L4449	336					+	1
L4450	337						0
L4451	338					+	1
L4453	340					+	1
L4454	341					+	1
L4455	342					+	1
L4457	344					+	1
L4458	345					+	1
L4459	346					+	1
L4461	348						0
L4462	349						0
L4463	350					+	1
L4464	351						0
L4465	352						0
L4466	353						0
L4467	354					+	1
L4469	356					+	1
L4470	357					+	1
L4471	358				+		1
L4473	360						0
L4474	361						0
L4475	362					+	1
L4476	363			+			1
L4477	364	+				+	2
L4478	365					+	1
L4479	366					+	1
L4481	368					+	1
L4482	369					+	1
L4483	370					+	1
L4485	372					+	1
L4486	373						0
L4487	374					+	1
L4488	375			+		+	2

VITA

Name:	Leslie Deanne Gilbert
Address:	Department of Marine Sciences Texas A&M University at Galveston c/o Dr. Robin Brinkmeyer 5007 Avenue U, Galveston, TX 77551
Email Address:	leslie.gilbert.07@tamu.edu
Education:	B.S., Marine Biology, Texas A&M University, 2007 M.S., Wildlife and Fisheries Sciences, Texas A&M University, 2010